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The germ cell – the mother of all stem cells

PETER J. DONOVAN*

Cell Biology of Development and Differentiation Group, ABL-Basic Research Program, NCI-FCRDC, Frederick MD, USA

ABSTRACT The germline, uniquely amongst the lineages of the embryo, carries the genome from generation to generation and is therefore the only lineage which retains true developmental totipotency. Paradoxically, when mouse primordial germ cells (PGCs) are introduced into a host blastocyst, they do not contribute to either the germline or the soma, suggesting that they are restricted in developmental potency. Conversely, *in vivo* PGCs give rise to embryonal carcinoma (EC) cells, the pluripotent stem cells of teratomas, benign tumors containing derivatives of the three primary germ layers. Similarly, PGCs can be converted *in vitro* into embryonic germ (EG) cells, pluripotent stem cells capable of giving rise to somatic and germline chimeras. The ability of PGCs to form EC cells *in vivo* and EG cells *in vitro* suggests that developmental potency of PGCs is regulateable. The molecular mechanisms controlling PGC growth and differentiation are gradually being elucidated through the characterization of sterile mutants and through the use of *in vitro* culture systems. Understanding how a PGC can give rise to a pluripotent stem cell could give significant insights into the regulation of developmental totipotency as well as having important implications for male fertility and the etiology of testicular cancer.

KEY WORDS: *PGC, mouse, embryo, EG cells, teratoma*

Introduction

PGCs are the embryonic precursors of the gametes of the mature adult animal and, collectively with the gametes, are cells of the germline or germ cell lineage. The germline, uniquely among the cell lineages of the embryo, will carry the genome on to the next generation while the somatic cell lineages will form the body of the animal and will eventually die. The ability of germ cells (but not other cells of the body) to repeat the process of embryonic development over and over again is unique and gives rise to the idea that germ cells are potentially immortal. Therefore, the analysis of PGC development is central to our understanding of the regulation of developmental totipotency in vertebrates. The development of pluripotent stem cells, termed embryonic germ cells or EG cells (similar to embryonic stem (ES) cells), directly from PGCs in culture, while an important technological advance, also has important implications for our understanding of developmental totipotency. It also seems likely that these studies will be relevant to our understanding of the etiology of germ cell-derived tumors, teratomas and teratocarcinomas.

In mammals, cytoplasmic determinants present in the egg and responsible for determination of the germline, such as those found in *Drosophila* and *Xenopus*, have not been identified and PGCs are thought to be determined relatively late in development. Individual blastomeres of cleavage-stage mouse embryos retain developmental totipotency, suggesting that mammalian eggs are unlikely to contain cytoplasmic germline determinants. In mice and a number of other vertebrates species, PGCs have been classically identified by alkaline phosphatase histochemistry. Mouse PGCs express a specific izozyme of alkaline phosphatase, tissue non-specific alkaline phosphatase (TNAP) on their cell surface (MacGregor *et al.*, 1995). TNAP has been used as the classical marker by which PGCs have been traced in mammalian embryos. This has allowed both the number of PGCs and their location to be accurately determined during embryonic development. In mice, PGCs are first identified by TNAP staining in the 7.0 days post coitum (dpc) embryo when a small number of PGCs (8-10) are identified at the caudal end of the primitive streak in the extraembryonic mesoderm. Fate mapping studies demonstrate that these cells emerge from a small number of progenitors that arise in the most proximal part of the presumptive extraembryonic mesoderm before gastrulation. At this time of development, these progenitors are not yet restricted to a germline fate. In other words, the progenitors of the PGCs can also give rise to other cell lineages. The PGCs progenitors are scattered in a ring in the epiblast close

Abbreviations used in this paper: PGC, primordial germ cell; TNAP, alkaline phosphatase; dpc, days post coitus; EG, embryonic germ; EC, embryonal carcinoma.

***Address for reprints:** Kimmel Cancer Center, Thomas Jefferson University, 233 South 10th street, BLSB 706, Philadelphia, PA 19107, USA. FAX: 215 923 4153. e-mail: pdonovan@lac.jci.tju.edu

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to the extraembryonic ectoderm before the formation of the primitive streak. The PGC progenitors are then translocated, as part of the expanding epiblast, to the posterior end of the primitive streak as the epiblast expands towards and through the primitive streak early in gastrulation. During the subsequent few days of embryonic development (7.0 dpc-8.5 dpc), the PGCs are located in an extraembryonic location at the caudal end of the primitive streak near the allantois. At 8.5 dpc TNAP staining reveals that there are approximately 50-100 PGCs in the embryo. At present the reason why PGCs are located outside of the embryo proper at this time of development is not understood. This small population of cells is carried into the embryo during the formation (invagination) of the gut and by 9 dpc PGCs are found within the hindgut. Over the next two to three days of development, PGCs migrate out of the hindgut and up the hindgut mesentery that joins the hindgut to the dorsal body wall. They migrate toward two thickened ridges of tissues that lie on either side of the mesentery, on the dorsal body wall. These are the so-called genital ridges, the gonad anlage, which upon further development will form the gonads of the adult animal. During the course of migration and for a couple of days after they reach the gonad, PGCs will proliferate to give rise to the population of cells that will enter into meiosis. So the 50 to 100 cells that are first identified in the embryo at 7 dpc give rise to approximately 35,000 cells in the embryonic gonad at the 13th day of development (reviewed in McLaren, 1981; Wylie *et al.,* 1985). The reduction in the numbers of TNAP-positive cells in sterile mice confirms the identity of the TNAP-positive cells as PGCs. Changes in PGC surface antigen expression also occur during this period (Donovan *et al.*, 1986). Many of these cell surface antigens are carbohydrate differentiation antigens and represent post-translational modifications of glycolipids and glycoproteins. Perhaps the best studied of these antigens is the stage-specific embryonic antigen-1 (SSEA-1) (Solter and Knowles, 1978) which represents a fucosylated lactosamine modification of glycoprotein and glycolipid cores. The SSEA-1 antigen is expressed on the PGC surface from 8.5 dpc, through the period of migration, and is lost after PGCs colonize the gonad anlagen (Fox *et al.,* 1981; Donovan, *et al.*, 1986). Analysis of the role of the SSEA-1 antigen in preimplantation development led to the hypothesis that these carbohydrate residues might be involved in compaction and cell adhesion (Bird and Kimber, 1984). Similar studies on the role of the SSEA-1 antigen in PGC development also led to a similar conclusion, namely that the SSEA-1 antigen might be part of a PGC cell adhesion molecule (Donovan *et al.*, 1987). The functional significance of antigenic modulation during the development of PGCs remains unclear but the presence of these antigens on the surface of PGCs represent useful tools for the purification of PGCs from embryonic gonads and their identification in culture (Donovan, *et al.,* 1986; McCarrey *et al.,* 1987).

The fate of germ cells in the gonad from then on depends in large part on the sex of the embryo. Male PGCs will cease proliferation and enter a mitotic arrest, and will remain in that state until a few days after birth. At this time they resume mitosis, giving rise to the mitotic stem cell of the adult animal, the spermatogonia. It is these cells that, at puberty, will undergo meiosis and spermiogenesis to give rise to mature sperm. In female embryos the PGCs transit directly from a mitotic division into a meiotic division and then arrest at meiotic prophase. From then until birth there is a tremendous loss of female germ cells, a process known as atresia. After birth female germ cells will re-enter meiosis in waves, pass through metaphase I of meiosis I and then arrest at metaphase II of meiosis. These germ cells are now fully formed oogonia (see McLaren 1981; Wylie *et al.,* 1985, for reviews).

Teratomas and the regulation of PGC differentiation

The stability of PGC differentiation in the gonad may be altered when PGCs give rise to benign tumors (teratomas) or malignant tumors (teratocarcinomas). Teratomas develop as small clumps of rapidly dividing undifferentiated cells, termed embryonal carcinoma or EC cells, within the seminiferous tubules of male fetuses at 15.5 dpc (Stevens, 1966). After enlargement, the tumors resemble 5- or 6-day embryos. Finally, cells in the tumor differentiate, giving rise to cell types derived from the three primary embryonic germ layers: endoderm, ectoderm, and mesoderm. Genetic, morphological, and immunological evidence suggest that these tumors are derived from PGCs (Stevens, 1967). For example, EC cells resemble PGCs in that they continue to express TNAP and the SSEA—1 antigen (Martin and Lock, 1983). The ability of PGCs to give rise to EC cells and teratomas suggests that they retain some developmental pluripotency. When genital ridges were isolated from 11.5-dpc embryos and transplanted into the testis capsule of adult syngeneic hosts, they generated to tumors with high efficiency. The ability of genital ridges to give rise to teratomas declined with developmental age such that no tumors were derived from grafts of 14.5 dpc gonads (Stevens, 1966). These results infer that a dramatic change in PGC potential occurs after colonization of the gonad anlagen (Stevens, 1967). Whether this change reflects an alteration in the pluripotency of the germ line or merely the change in proliferative activity (and, therefore, the ability to give rise to tumors) of PGCs at this time is unknown.

In the mouse, the developmental potential of cells has classically been tested by analyzing the fate of genetically marked cells in aggregation or blastocyst-injection chimeras. Cells of the epiblast, from which PGCs are thought to derive, were found to be restricted in developmental potential when assayed in this way. The conclusion drawn from these experiments is that PGCs are also restricted in their developmental potency (McLaren, 1981). However, since the fertilized oocyte is totipotent, PGCs must have totipotency restored to them at some stage in development. It has been suggested the totipotency is restored to the female germ line by some process linked to reactivation of the silent X chromosome. When chimeras were formed by injection of PGCs isolated from 10.5 dpc embryos into blastocysts, no evidence was found for contributions to the germ line or the soma in adult animals (reviewed by Donovan, 1993). This suggests that PGCs are restricted in their developmental potential at this stage of development. The ability of EC cells to form chimeras was first tested by Ralph Brinster (Brinster, 1974) and then by others (Papaioannou *et al.*, 1975; Illmensee and Mintz, 1976; Illmensee, 1978; Papaioannou, 1979). These studies unequivocally demonstrated that EC cells were developmental pluripotent. These data reveal an important paradox: PGCs appear to be restricted in developmental potency while the EC cells derived from them are developmentally pluripotent. Understanding this paradox could be particularly informative as to the molecular mechanisms regulating developmental potency in mammals. Of course, whether PGCs are really developmentally restricted is an important question. Is it possible that the inability of PGCs to contribute to the soma and germline of

chimeras is simply due to their inability to survive or proliferate in the blastocyst? This question could be answered by nuclear transplantation experiments but these experiments are unlikely to be easily accomplished. Nevertheless, even if the PGC nucleus is not strictly speaking restricted in developmental potential, it is important to understand the transition from a PGC to an EC cell. What are the factors that control PGC growth in the embryonic gonad and what are the factors that regulate PGC differentiation? Some of the molecules regulating PGC survival, proliferation and differentiation have been elucidated and these studies have led to the development of *in vitro* culture conditions which may mimic the process of teratocarcinogenesis *in vivo*.

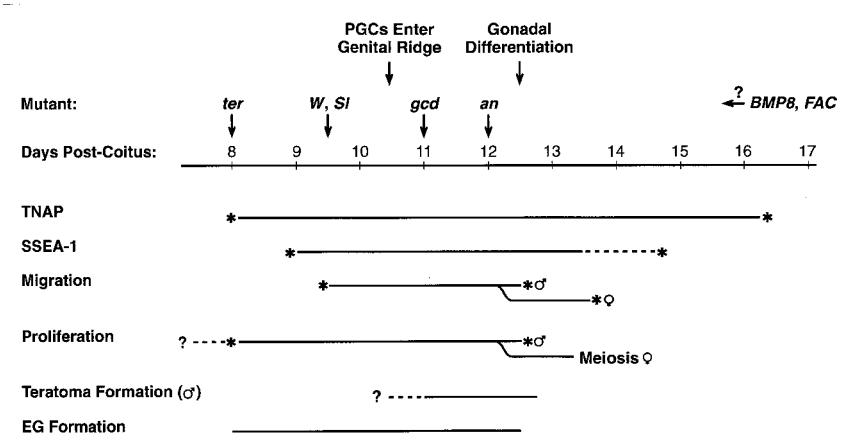

Fig. 1. Characteristics of PGC development in the mouse embryo. *The solid line represents the stage of embryonic development measured as days post coitus (dpc), where the identification of a vaginal plug is taken as 0.5 dpc. The lines below reflect the expression of markers such as TNAP or SSEA-1 or of phenotypic traits such as migration or the ability of PGCs to give rise to teratomas in vivo or EG cells in vitro. The symbols above the line denote sterile mutants (ter, W, Sl, an, and gcd) and are denoted at the time at which these mutations act to affect PGC development.*

Regulation of PGC growth and differentiation

The characterization of sterile mouse mutants and the use of *in vitro* culture systems have helped clarify some of the factors that regulate PGC proliferation and differentiation (Donovan, 1993; Matsui, 1997). In mice there are four well known mutants that, when homozygous, cause sterility and identify genes that act during the embryonic period of germline development. These include the semi-dominant *Steel (Sl)* and *Dominant White Spotting (W)* mutants and the recessive *Hertwig's anemia (an)* and *germ cell deficient (gcd)* mutants. The molecular bases of the *W* and *Sl* mutations have been determined and have been described extensively elsewhere. Briefly, the *W* locus encodes a receptor tyrosine kinase (c-kit) (Chabot *et al.,* 1988; Geissler *et al.,* 1988) and the *Sl* locus encodes its ligand, variously called Kit-ligand, mast cell growth factor, stem cell factor or Steel factor (SLF) (Copeland *et al.,* 1990; Zsebo *et al.,* 1990).

The c-kit receptor is characterized in part by having a split tyrosine kinase domain and five immunoglobulin-like repeats in the extracellular domain (Yarden *et al.,* 1987; Qui *et al.,* 1988). Mutations that delete the *c-kit* gene are lethal when homozygous but show the mildest phenotype in the heterozygous condition. Other mutations, such as the original *W* mutation, result from partial deletions that truncate the c-kit kinase. These mutations are strong semi-dominant mutations that, when homozygous, cause a range in phenotype from viability to early post-natal lethality. That these mutations are semi-dominant is explained by the requirement for c-kit dimerization for activation of kinase activity and for signaling. The formation of a heterodimer between a wildtype protein and a truncated protein could result in altered ligand-binding or altered kinase activation, either event likely to result in impaired receptor activation. The W^{42} mutation, one of the most dominant *W* mutations, results from a point mutation in the c-kit kinase domain. This produces a receptor which is an inactive kinase. W^{42}/W^{42} mice lack germ cells demonstrating the requirement for this signaling pathway in germ cell development. Such animals are also severely anemic and lack coat melanocytes, pointing to the requirement for this signaling pathway in both hemopoietic and melanoblast development (Tan *et al.,* 1990).

In theory the c-kit signaling pathway could act in a number of different ways to affect germ cell development. It could effect PGC survival, proliferation, migration or differentiation. Increasing evidence suggests that SLF acts as a survival factor for PGCs that, in concert with other factors, stimulates germ cell proliferation (Dolci *et al.,* 1991; Godin *et al.,* 1991; Matsui *et al.,* 1991; Pesce *et al.,* 1993). SLF is a transmembrane growth factor that is produced in two forms, generated by alternate splicing, one of which has a proteolytic cleavage site in the ectodomain and can be cleaved to give rise to a soluble growth factor. The other form lacks the proteolytic cleavage site and remains as a membrane-associated factor. The large collection of mutations at the *Sl* locus contains two that are particularly informative about the structure and function of the different domains of SLF. These are the Sl^{d} and Sl^{17H} mutations, both of which have been characterized at the molecular level. The Sl^{d} mutation, which arose spontaneously, results from an intragenic deletion. This deletion removes sequences encoding the cytoplasmic tail and transmembrane region of SLF (Brannan *et al.,* 1991; Flanagan *et al.,* 1991). The Sl^{d} allele therefore encodes a soluble factor. Sl^{d} homozygotes are viable (on some genetic backgrounds) suggesting that this soluble factor is sufficient to support development of the hemopoietic lineages. However, these animals are sterile, demonstrating that soluble forms of SLF are insufficient for development of the germline. When transmembrane and soluble forms of SLF were compared for their effect on cultured PGCs, a clear distinction was observed. PGCs do not survive for even 24 h when cultured on feeder layers of cells that do not express SLF (Dolci *et al.,* 1991). These feeder cells can then be transfected with vectors capable of expressing different forms of SLF. When cells are transfected with constructs expressing full-length, transmembrane forms of SLF, they efficiently support PGC survival for 3 to 5 days depending on the transfection efficiency (Dolci *et al.,* 1991). On the other hand, when these cells are transfected with constructs expressing a soluble form of SLF, equivalent to that encoded by the Sl^{d} allele, then PGCs can survive for only 24 h. When a soluble, recombinant SLF is added to the medium, it improves initial survival but is unable to effect long-term

survival (Dolci *et al.,* 1991). Similar results are seen when PGCs are cultured on bone marrow stromal cell lines derived from wildtype or *Sl/Sl*^{d} animals (Dolci *et al.,* 1991; Matsui *et al.,* 1991). These data suggest that long-term PGC survival requires a membrane-bound form of SLF. This could explain the sterility seen in *Sl*^{d} animals and would also provide an exquisite mechanism for controlling PGC survival in the embryo. Restricted expression of transmembrane forms of SLF could control both where PGCs can adhere (and, therefore, migrate) as well as where they can survive. PGCs that migrate off the normal pathway would be expected to die through programmed cell death. Consistent with this idea, PGC migration is abnormal in mice expressing only soluble forms of SLF and in a *W* mutant, *W*^{e} (McCoshen and McCallion, 1975; Buehr *et al*., 1993), and, moreover, anti-c-kit antibodies block PGC adhesion in culture (Pesce *et al.,* 1997).

The second *Sl* mutation that is informative as to the role of SLF in early PGC development is *Sl*^{17H}. This mutation arose by ENU-mutagenesis and results in the production of a form of SLF with an abnormal cytoplasmic tail (Brannan *et al.,* 1992). PGC numbers are affected in homozygous embryos of both sexes, but adult females have enough gonia to produce normal-sized litters. In males, the spermatogonia undergo one round of spermatogenesis, but then fail to undergo self-renewal. The role of the cytoplasmic tail of SLF remains uncertain but its possible functions include: stabilization of the factor in the membrane, growth factor dimerization, regulation of ectodomain cleavage, and finally intracellular signaling. Further studies on the role of the cytoplasmic tail of SLF will undoubtedly yield important information on the role of this growth factor in the development of the mammalian germline.

The development of systems for culturing PGCs *in vitro* were first aimed at studying PGC migration (Blandau *et al*., 1963) but subsequent studies, including those of Ralph Brinster (which influenced our own efforts), developed more sophisticated techniques for maintaining PGCs and allowing them to proliferate *in vitro* (Brinster and Harstad, 1977; De Felici and McLaren, 1982,1983; Donovan *et al*., 1986; Stott and Wylie, 1986). *In vitro* studies have now identified several factors other than SLF which can modulate PGC survival and proliferation. Two of the factors identified, leukemia inhibitory factor (LIF) and basic fibroblast growth factor (bFGF), are relevant to the development of teratomas and teratocarcinomas. LIF is a cytokine related in secondary structure and genomic organization to Interleukin-6 (IL-6), Oncostatin M (OSM) and Ciliary Neurotrophic Factor (CNTF). The LIF receptor has been characterized and comprises a low-affinity, LIF-binding subunit (LIFR), and a second transmembrane molecule, gp130, which was originally identified as the IL-6 signal transducer. Heterodimerization of LIFR and gp130 is required for activation of the LIF receptor and is accompanied by tyrosine phosphorylation of both subunits. LIFR and gp130 both act as components of bipartite receptors for multiple cytokines. The LIFR/gp130 complex also functions as a high affinity receptor for OSM and, together with a third subunit, is a high affinity receptor for CNTF. How does LIF affect PGC growth? Like SLF, LIF acts to promote PGC survival and together with SLF stimulates PGC proliferation (De Felici and Dolci, 1991; Matsui *et al*., 1991; Resnick *et al*., 1992; Dolci *et al.,* 1993). However, the role of LIF in PGC development remains unclear since LIF is not detectable in the embryonic gonad and LIF-deficient mice have no observable defect in PGC numbers and produce viable gametes (Cheng, Stewart and Donovan, unpublished observations). Other members of the LIF cytokine family, such as OSM can stimulate PGC proliferation *in vitro* and may do so *in vivo* (Cheng *et al*., 1994). Although LIF may not be the physiological ligand for PGCs, the importance of this cytokine family in PGC development is clearly demonstrated by the ability of anti-gp130 antibodies to block PGC survival (Koshimizu *et al*., 1996) and by the severe deficiency in PGC numbers in mice lacking the signaling component of the LIF receptor, gp130 (Yoshida *et al.,* 1996; Taga and Kishimoto, personal communication). Taken together these data suggest that a member of the LIF cytokine family is likely to be an important regulator of PGC growth *in vivo*. Intriguingly, LIF and the related cytokine OSM, map close to the *gcd* locus on mouse Chromosome 11 (Duncan *et al.,* 1995). Whether *gcd* represents a mutation in structural or regulatory elements of the LIF or OSM genes will be important to determine.

Because of the complexity of the FGF family and the complexity of their receptors, the role of FGFs remains less clear. It will be important to determine which FGF receptor(s) PGCs express and which member(s) of the FGF family are involved in PGC development. At this time, over a dozen members of the FGF family have been identified. Of these factors, FGF4 (K-FGF) seems the best candidate for the physiological ligand for PGCs since it is expressed in the primitive streak at around the time when PGCs are moving through the embryo (Niswander and Martin, 1992; Drucker and Goldfarb, 1993). Recently the *Ter* locus, the major determinant of teratoma and teratocarcinoma formation in mice, has been localized on mouse Chromosome 18 close to the acidic FGF gene (aFGF or FGF1). This data suggests that aFGF may be a regulator of PGC proliferation and differentiation (Asada *et al*., 1994; Sakurai *et al.,* 1994).

Currently a large number of other potential PGC growth regulators have been identified (Table 1). However, the role of some of the factors identified using *in vitro* culture systems must be viewed cautiously since they may not act directly on the PGCs but rather through surrounding feeder cells or accompanying embryonic somatic cells. Understanding the physiological role of many of

TABLE 1

PGC GROWTH REGULATORS

Factor	Reference
Steel Factor	Dolci *et al.,* 1991; Matsui *et al.,* 1991; Godin *et al.,* 1991
Leukemia Inhibitory Factor	Matsui *et al.,* 1991; Dolci *et al.,* 1993; Resnick *et al.,* 1992
basic Fibroblast Growth Factor	Matsui *et al.,* 1992; Resnick *et al.,* 1992
Tumor Necrosis Factor-a	Kawase *et al.,* 1994
Interleukin-4	Cooke *et al.,* 1996
Growth Arrest Gene-6	Matsubara *et al.,* 1996
Pituitary adenylate cyclase-activating peptide	Pesce *et al.,* 1996
Retinoic acid	Koshimizu *et al.,* 1995

these factors may require cell type-specific gene targeting in PGCs or gonadal somatic cells using a system such as Cre-Lox.

Nevertheless, PGCs can be effectively immortalized in the presence of a cocktail of growth factors: SCF, LIF and basic Fibroblast Growth Factor (bFGF), and a confluent feeder layer of STO (or Sl220) cells (Matsui *et al.,* 1992; Resnick *et al.,* 1992). In these conditions, PGC numbers increase dramatically during the first few days of culture and continue to do so until well after the time they would normally differentiate or die *in vitro* (Donovan *et al.*, 1986). Initially, the PGCs form small colonies of approximately 8-10 cells that expand as a monolayer on top of the feeder layer. By about 7-8 days of culture, these colonies begin to form multilayered clumps. By 9 days, these clumps are clearly visible to the naked eye. When the clumps of cells are dispersed by trypsinization and re-plated onto fresh feeder layers, they form new colonies that can be passaged indefinitely (Matsui *et al.,* 1992; Resnick *et al.,* 1992). The resulting cells, termed EG cells, are similar to pluripotent ES cells in many respects. They continue to express alkaline phosphatase as well as the SSEA-1 antigen (Matsui *et al.*, 1992; Resnick *et al.*, 1992). EG cells form embryoid bodies in culture, give rise to teratomas when introduced into histocompatible animals, and form germline chimeras when introduced into a host blastocyst (Matsui *et al.,* 1992; Labosky *et al.,* 1994a,b; Stewart *et al.,* 1994; L. Jackson-Grusby and R. Jaenisch, personal communication; U. Klemm and R. Mulligan, personal communication; our own unpublished observations). Indeed, Balb/c-derived EG cell lines have been used to create IL-4-deficient mice by homologous recombination (U. Klemm and R. Mulligan, personal communication). The parallels between the development of EC cells *in vivo* and the formation of EG cells *in vitro* are obvious. In both situations PGC give rise to a cell type that grows as a stem cell which, upon appropriate stimulation, can differentiate into derivatives of the three primary germ layers. Is the process of EG cell derivation *in vitro* related to the formation of EC cells *in vivo*?

How does a combination of growth factors affect the growth and developmental potential of PGCs? Once in the gonad PGCs proliferate for a period of time before they differentiate to form gonia. In the male, the PGCs simply enter a quiescent phase (arrested in the G_1 phase of the cell cycle) in which they do not divide (Hilscher *et al.,* 1974). This arrest is accompanied by an observed down regulation of c-kit mRNA levels. Again this suggests that the proliferative potential of PGCs might be linked to the levels of c-kit in the cell. We proposed a model for both the normal regulation of c-kit levels in the embryo and the aberrant proliferation of PGCs seen in the early stages of teratocarcinogenesis. In this model, the level of c-kit in PGCs might be regulated by exposure to another growth factor, namely a fibroblast growth factor (FGF). PGCs migrating through the primitive streak might be exposed to FGF4, which could upregulate c-kit levels in PGCs, converting a c-kit-low PGC to a c-kit-high PGC. We have also suggested that this process would start a c-kit-dependent clock that would also time the length of PGC proliferation in the embryo. A similar model has been proposed for the regulation of proliferation of O-2A progenitors in the developing optic nerve (McKinnon, *et al.,* 1990). An obvious extension of this model is that exposure of PGCs to an FGF at other times might upregulate c-kit levels and cause continued proliferation of PGCs. In this regard it is interesting to note that one candidate for the *Ter* mutant is another member of the FGF family, acidic FGF (Asada *et al.,* 1994; Sakurai *et al.*, 1994). Perhaps c-kit signaling can, in concert with other signaling pathways, control PGC proliferation and, when this pathway is inappropriately activated, lead to continued proliferation of PGCs. In the abnormal condition (teratoma and teratocarcinoma), PGCs might fail to block at G_1 and continue through additional rounds of cell division. The ability of the c-kit protooncogene to cause cellular transformation has been described in the hemopoietic system where activated forms of c-kit have been identified in mast cell-derived tumors (Furitsu *et al.,* 1993; Longley *et al.,* 1996; Piao *et al.,* 1996). It is also noteworthy that while c-kit levels are very low or undetectable in human non-seminoma and teratoma, c-kit levels are high in carcinoma *in situ* (CIS) and seminoma (see for example Rajpert-De Meyts and Skakkebaek, 1994). These data suggest that PGC transformation leading to CIS in humans might involve inappropriate activation of the c-kit signaling pathway. This hypothesis is testable by overexpression by c-kit in PGCs in transgenic mice using the Oct 4 promoter (Yeom *et al.*, 1996).

Perspectives

Historically the germline has been one of the most studied lineages in the vertebrate embryo. The molecular mechanisms regulating PGC development are gradually being elucidated in a variety of species including vertebrates. Understanding the mechanisms by which PGCs are formed in the embryo and by which they regulate developmental totipotency is one of the most important questions in modern developmental biology. Moreover, factors which affect PGC development are likely to be important in determining fertility and in influencing gonadal tumor development in males and females. Advances in this area of research may come from a variety of avenues. Firstly, characterization of existing sterile mouse mutants will identify some of the genes involved in PGC development. The *gcd* and *an* mutants in particular may be particularly informative as to early PGC development. The *gcd* mutant arose by insertional mutagenesis of a 8.0 kilobase goat fetal β-globin transgene on mouse Chromosome 11 (Pellas *et al.*, 1991). PGCs in *gcd* animals are affected as early as 11.5 dpc but no defect is observed in the somatic cell lineages. Both male and female adult mice are infertile. Because of the effect of the *gcd* mutation on ovarian development and function, this mutant has been proposed as a model for premature ovarian failure (Duncan *et al.,* 1993). The localization of the transgene insertion on mouse chromosome 11 near the LIF and OSM genes is interesting given the effect of these factors on PGC survival and proliferation (Duncan *et al.*, 1995). However, Southern blot analysis revealed no gross rearrangements in these genes in *gcd* mice (Duncan *et al.*, 1995). There is a clear precedent, however, for mutations affecting, not the coding region of genes, but of their regulatory elements. For example, large chromosomal deletions on mouse Chromosome 10 disrupt the regulatory elements of the Steel locus. The Sl^{Panda} and $Sl^{contrasted}$ alleles have severe effects on PGC development without affecting the structural elements of the gene (Bedell *et al.*, 1995). *gcd* could, therefore, represent a disruption of regulatory elements controlling the expression of the LIF and OSM genes but this remains to be determined. The *an* mutation, which causes both macrocytic anemia and reduced germ cell number, was identified in the F2 offspring of a heavily irradiated male mouse (Hertwig, 1942). Adult mice of both sexes were found to be usually (but not always) sterile. The differences in gonadal development between

adult *an/an* mice and their normal littermates were found to be present at birth, suggesting that this mutation affects PGC development. Subsequent analysis of PGC development in *an/an* embryos demonstrated that the first defects in PGC numbers could be detected at 12.5 dpc (Russell *et al.*, 1985). The ability to rapidly determine the chromosomal localization of new genes will continue to provide important new candidates for genetic loci affecting fertility and teratocarcinogenesis in mice and humans. Furthermore, the ability to rapidly construct YAC and BAC contigs of a region and to isolate candidate genes means that many loci affecting fertility and teratocarcinogenesis will likely be molecularly cloned in the near future, even if a candidate gene does not already exist. Also a large number of knockout mice (generated by homologous recombination in embryonic stem cells) have been produced over the last few years. Many of these mice have been found to have defects in germline development. For example, targeted disruption of the BMP8 and fanconia anemia-*c (Fac)* genes have revealed important roles for these genes in germline development (Whitney *et al* , 1996; Zhao *et al.*, 1996). This has provided a windfall of information about the molecular regulation of germline development in mammals and is likely to continue to do so for some time to come. However, some important information will be missed if it is assumed that knockout mice, if fertile, have no defects in germline development. The plasticity of the germline can make up for deficiencies during PGC development. Some *Sl* mutants have severe deficiencies in PGC development but are fully fertile because spermatogonial proliferation compensates for the reduced numbers of PGCs (Bedell *et al.*, 1995). It will be important, therefore, to examine germline development in many "knockout" mice even if an obvious reproductive defect is not apparent. Secondly, advances in research concerning germline development in vertebrates are likely to come as a result of new genomic and high throughput sequencing technologies. Over the last few years high throughput sequencing of expressed sequence tags (ESTs) has provided a valuable insight into gene expression in a variety of cell types. The application of this technology to the analysis of germline development is already underway and is likely to generate a wealth of information on the genes expressed in, and regulating the development of, germ cells. Thirdly, the analysis of germline development in *Drosophila* and *Caenorhabditis* will likely continue to provide important clues as to the mechanisms regulating germline development in vertebrates. Significant information regarding the regulation of the meiotic cell cycle may also come from studies on meiosis in yeast. Analysis of PGC development in the Zebrafish would undoubtedly advance our understanding of germline development in the vertebrates. This experimental organism has a combination of the superb genetics of flies and worms and the experimental manipulability of *Xenopus*. Large scale screens for mutants affecting germline development in this vertebrate might yield important new clues to the molecular mechanisms regulating germline development in mice and men.

Fourth, and finally, the characterization of genetic loci affecting teratocarcinogenesis in mice and humans will be a key to determining the molecules regulating the control of PGC development and differentiation. Some of the genetic loci affecting teratocarcinogenesis in mice have been identified and include the agouti yellow allele (A^y), situs inversus (iv), the *Sl* gene, and a mutation designated ter (for teratoma). When this mutation was introduced onto the 129/Sv background, the resulting stock (129/Sv-ter) developed spontaneous testicular teratomas in 33% of the males by comparison with 3-5% in control animals. Interestingly, the *ter* gene has a drastic effect on PGC numbers which is first detected at 8.0 dpc (Sakurai *et al.*, 1995). In this respect, the ter mutation resembles some of the *Sl* mutations which also affect PGC survival. Why genes that affect PGC survival should have such a drastic effect on the incidence of testicular teratoma is an interesting question.

Summary

The molecular mechanisms regulating the development of the mammalian germline are gradually being determined. Some of the growth factors regulating the development of PGCs in the mouse embryo have been identified through classical genetics and through the use of cell culture systems. However, growth control in the germline is likely to be complex and it is expected that other factors yet to be identified play important roles in regulating PGC growth and differentiation. *In vivo* and *in vitro* PGCs can give rise to pluripotent stem cells capable of giving rise to cells of multiple lineages. Understanding the mechanisms of PGC "transformation" into a pluripotent stem cell would be an important technical advance as well as giving a critical insight into the regulation of developmental totipotency. These studies will also be important for understanding the etiology of human testicular cancer.

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